Reference: *Biol. Bull.* **184:** 115–124. (April, 1993)

Ooplasmic Segregation in the Medaka (*Oryzias latipes*) Egg

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Abstract. Using time-lapse video microscopy, we found that ooplasmic inclusions in the fertilized medaka egg displayed two types of movement during ooplasmic segregation. The first manifested itself as the movement of many inclusions (diameter = 1.5–11 μm) toward the animal pole at about 2.2 μm min^{-1}; this type of movement appeared to be streaming. The second type of movement was faster (about 44 μm min^{-1}) and saltatory; inclusions displaying this type of movement were smaller (diameter $\leq$ 1.0 μm) and moved toward the vegetal pole. The movement of oil droplets toward the vegetal pole of the egg may represent a third type of motion. All these movements began only after a strong contraction of the ooplasm toward the animal pole, which at 25°C began 10–12 min after fertilization and <3 min after formation of the second polar body.

In eggs treated with microtubule poisons—colchicine, colcemid, or nocodazole—oil droplets did not move toward the vegetal pole, saltatory motion toward the vegetal pole was absent, and the growth of the blastodisc was slowed. Eggs treated with β-lumicolchicine, an inactive derivative of colchicine, showed normal movements. Colchicine, while not inhibiting formation of the second polar body, did inhibit pronuclear migration. These results suggest that microtubules are involved in the movement of some ooplasmic inclusions, including oil droplets, toward the vegetal pole; the movement of ooplasmic inclusions toward the animal pole; and pronuclear migration.

Introduction

Eggs of many animal species display a remarkable variety of movements soon after they are fertilized. Many of these movements, known collectively as ooplasmic segregation, are important for the rearrangement of egg cytoplasm during the minutes and hours following fertilization. In some animals, amphibians and ascidians for example, these movements lead to cytoplasmic localization of morphogenetic determinants, which are subsequently segregated to specific cells during cleavage and ultimately affect gene expression in the cells that incorporate them (Reverberi, 1971; Davidson, 1976; Illmensee *et al.*, 1976; Jeffery, 1984; Speksnijder *et al.*, 1990a).

In contrast to a relatively detailed understanding of ooplasmic segregation in eggs of ascidians, annelids, and amphibians (Vacquier, 1981), relatively little is known about it in fish eggs. Except for Roosen-Runge's (1938) classic study in which time-lapse cinemicrography was used to monitor ooplasmic segregation in the zebrafish egg (*Brachydanio rerio*), there have been no published reports of the use of time-lapse microscopy to monitor segregation in a fish egg. Given the increasing use of fish embryos as model systems in the study of development (Kimmel, 1989; Powers, 1989; Kimmel *et al.*, 1990; Schindler, 1991), it is important to examine segregation in this group of organisms more closely.

Microtubules are required for ooplasmic movements in several taxa of animals, including amphibians (Wakahara, 1989; Houliston and Elinson, 1991; Peter *et al.*, 1991), ascidians (Zalokar, 1974; Sawada, 1988; Sawada and Schatten, 1989), and annelids (Eckberg, 1981; Shimizu, 1982; Astrow *et al.*, 1989). Microtubule poisons—drugs that block either assembly or disassembly of microtubules—have been useful tools in these studies (Zalokar, 1974; Eckberg, 1981; Shimizu, 1982; Astrow *et al.*, 1989; Sawada and Schatten, 1989), in which a role for microtubules is presumed when a particular movement is inhibited by one or more of these poisons. Because these poisons can have cytotoxic effects unrelated to their effects on microtubules, many studies have compared the

Received 18 May 1992; accepted 25 January 1993.

effects of more than one such class of these poisons and, also, have used as controls chemically similar derivatives that have low affinity for tubulin, the protein subunit of microtubules. For example, β-lumicolchicine, a derivative of colchicine (Wilson and Friedkin, 1967), can be used as a control for colchicine (Sabnis, 1981; Achler *et al.*, 1989; Peter *et al.*, 1991).

We have studied ooplasmic segregation in the egg of the medaka (*Oryzias latipes*). This large (diameter = 1.2 mm) clear egg, with its thin peripheral layer of ooplasm surrounding a central yolk vacuole, permits microscopic study of both the gross movements of ooplasm as well as the movement of ooplasmic inclusions. The objectives of the present study were (1) to describe the movements of ooplasmic inclusions, and (2) to monitor the effects on these movements of three drugs that block microtubule assembly (Wilson *et al.*, 1974; Dustin, 1984, ch. 5; Bray, 1992, p. 207).

A preliminary account of these findings has been published (Abraham and Fluck, 1991).

Materials and Methods

We removed gonads from breeding medaka (Yamamoto, 1967; Kirchen and West, 1976; Fluck, 1978) and placed them in a balanced saline solution (BSS: 111 m*M* NaCl; 5.37 m*M* KCl; 1.0 m*M* $CaCl_2$; 0.6 m*M* $MgSO_4$; HEPES, pH 7.3). Eggs were removed from the ovary, and the long chorionic fibers at the vegetal pole were removed with scissors. Eggs were fertilized in BSS (Yamamoto, 1967) and transferred to a microscope slide on which a cover glass was supported by four pillars of petroleum jelly. The cover glass was then pressed gently against the chorion to flatten a small region of the egg near its equator. Such flattening facilitated optical studies and also enabled us to roll the egg to achieve the desired orientation. All procedures were performed at room temperature (23–26°C in most experiments); in this temperature range, the first cell division begins after about 70 min. Because the rate of development varies inversely with temperature, we have reported the timing of events not only as "minutes after fertilization" but also as "normalized time" (t_n), where $t_n = 1.0$ is the time at which cytokinesis begins.

We monitored the movements of ooplasmic inclusions (or parcels) with time-lapse video microscopy, using a Nikon Optiphot or Diaphot microscope equipped with phase-contrast optics and connected through a Dage/MTI camera to a Panasonic NV-8050 time-lapse video cassette recorder. Using a 40× phase-contrast objective lens, we usually focused on a patch of ooplasm near the equator of the just-fertilized egg; with the Optiphot, the field of view was approximately 140 μm × 200 μm (total magnification = 882×) and with the Diaphot it was approximately 225 μm × 325 μm (total magnification = 542×). We measured the diameters of inclusions on the screen of the video monitor and corrected for scale. To measure the speed and direction of movement of the inclusions, we placed a transparent plastic sheet over the video monitor during playback and mapped the paths of randomly chosen inclusions at regular time intervals; the length of the time interval chosen, usually either 20 s or 40 s, depended on the average speed of the inclusions at the time.

To measure the thickness of the blastodisc, we viewed it in profile from the side, measured its thickness along the animal-vegetal axis, and corrected for scale. To measure the volume of the blastodisc, we viewed it in profile from the side and used an image analysis program (Microcomp Planar Morphometry, Southern Micro Instruments, Atlanta, Georgia) to measure three parameters (area, centroid x, and projected x) of one-half of the blastodisc, after drawing a line that bisected the image along the animal-vegetal axis. We then calculated its volume, using the following equation: volume = (2π) (area) (centroid x-projected x). The validity of this method was established by measuring standard objects. To measure the thickness of ooplasm elsewhere on the egg, we measured its thickness *en face* in the Z axis of the objective lens, using the presence of inclusions as a marker for ooplasm.

We used two methods to monitor the timing of the second meiotic division. In the first, we fixed eggs in 3.7% formaldehyde in BSS at regular intervals after fertilization. After rinsing away the fixative and staining the nuclei with Hoechst 33258 (10 μg ml^{-1} in BSS containing 1% Triton X-100), we examined the eggs with epifluorescence optics. In the second method, we microinjected Hoechst 33258 (100 μg ml^{-1}, dissolved in 50 m*M* K_2SO_4 and 10 m*M* HEPES, pH 7.2) into unfertilized eggs, injecting approximately 1.5 nl into the ooplasm at about 45° latitude from the animal pole. The method for microinjection was similar to that used by Fluck *et al.* (1991), except we used a high pressure microinjection system (Narashige IM-200). The injection process parthenogenetically activated the eggs, while Hoechst 33258 stained the maternal nuclear DNA. After placing these eggs between a coverglass and slide, we recorded movements of the ooplasmic inclusions, using a SIT camera coupled to the VCR, and monitored the second meiotic division by examining the eggs at regular intervals with epifluorescence optics. Room temperature was 19.5°C in this latter series of experiments.

Microtubule poisons

Stock solutions of colchicine (1 m*M* in BSS), β-lumicolchicine (1 m*M* in BSS), colcemid (0.35 m*M* in BSS), and nocodazole (2 mg ml^{-1}) in DMSO were diluted into BSS to make working solutions. Working solutions of nocodazole also contained 1% DMSO, which had no ap-

parent effect on the eggs. In preliminary experiments, we monitored the effects of several concentrations of each drug on the movement of oil droplets during ooplasmic segregation and found the minimum effective concentrations that disrupted their normal movement to be 100 μM colchicine, 0.35 μM colcemid, and 0.17 μM nocodazole; we used these concentrations in subsequent experiments. Eggs were generally incubated with the drugs for 1 h before fertilization and then fertilized in the same medium; however, in some experiments, eggs were incubated with the drugs for 1.5 h or 2 h before they were fertilized. In each experiment, we monitored one egg with time-lapse video microscopy and monitored an additional 15–20 eggs with a stereomicroscope.

To monitor the effect of colchicine on formation of the second polar body and migration of the pronuclei, control eggs (nine eggs from two females) and eggs treated with 100 μM colchicine (eight eggs from two females) were fixed in 3.7% formaldehyde in BSS at $t_n = 0.45$. After washing away the fixative, the eggs were stained with Hoechst 33258 and examined with epifluorescence optics.

Chemicals

Colcemid, colchicine, Hoechst 33258, β-lumicolchicine, and nocodazole were obtained from Sigma (St. Louis, Missouri) and formaldehyde from Electron Microscopy Sciences (Fort Washington, Pennsylvania).

Results

An early sign of egg activation was the cortical granule reaction, which spread as a wave from the animal pole to the vegetal pole in about 90 sec at 26°C, a result consistent with the time reported by Gilkey *et al.* (1978). After the cortical granule reaction, the ooplasm became relatively transparent (Fig. 1A), and several types of inclusions could be seen in it (Fig. 2). One class of inclusions were oil droplets, which with phase-contrast optics appeared as white spheres with diameters from <1.0–100 μm. That these spheres were oil droplets was confirmed by staining them with a lipophilic fluorescent dye, nile red (data not shown). About 1 min after the beginning of the cortical granule reaction, there was a strong contraction of the ooplasm, marked by the movement of all ooplasmic inclusions toward the animal pole; this fertilization contraction lasted about 1.5 min and thus was over within 2.5 min after fertilization, times also consistent with Gilkey *et al.* (1978). Our detailed study of the movement of ooplasmic inclusions began after the fertilization contraction.

At 10–12 min after fertilization (at $t_n \approx 0.16$ at 25°C), a second contraction occurred (Fig. 3), in which all ooplasmic inclusions, including oil droplets, again moved toward the animal pole. After this second contraction, most inclusions continued to move toward the animal pole; however, oil droplets (Fig. 1C–F) and some smaller inclusions began to move toward the vegetal pole. Accumulation of ooplasm at the animal pole and the movement of oil droplets and other inclusions toward the vegetal pole proceeded simultaneously for ≈70 min, at which time the blastodisc underwent its first division (Fig. 1F). By this time, there were fewer, larger oil droplets, a result of their fusion with each other during their movement toward the vegetal pole.

The timing of the second meiotic division was approximated by examining fixed eggs and was confirmed by injecting Hoechst 33258 into live eggs. At 19.5°C the second polar body formed by 13.8 ± 3.3 min ($\bar{X}$ ± S.D., n = 4; $t_n \approx 0.12$) after activation, and the second contraction began about 3 min later at 16.7 ± 1.2 min ($\bar{X}$ ± S.D., n = 6; $t_n \approx 0.15$; Fig. 2). In all cases, polar body formation preceded the second contraction.

In the following three sections, we describe (1) the streaming movement of inclusions toward the animal pole, (2) the saltatory movement of inclusions toward the vegetal pole, and (3) the movement of oil droplets toward the vegetal pole. The data presented in Figures 4 and 5 were collected from a single egg at $t_n \approx 0.43$. The movements seen in this egg were confirmed in 41 other eggs studied between the second contraction and the first cell division, and an additional 15 eggs were used to obtain the data summarized in Figure 2.

Streaming

Essentially all the inclusions in Figure 2 appeared to be streaming toward the animal pole. The diameters of these inclusions were in the range 1.5–11 μm, and they appeared to be distributed throughout the depth of the ooplasm. By "streaming" we mean that the movements of the individual inclusions did not appear to be independent of each other; in other words, all inclusions moved at nearly the same speed and in the same direction. The motion of three such inclusions, moving at about 1.5 μm min^{-1}, is summarized in Fig. 4A. Though this speed was typical of streaming motion (2.2 ± 0.8 μm min^{-1}, $\bar{X}$ ± S.D., n = 31 inclusions from 5 eggs), the speed sometimes increased to as high as 8.2 μm min^{-1} for periods lasting up to 10 min and sometimes decreased to near zero for periods lasting up to 12 min.

Saltatory movement

The circled inclusion in Figure 2 is one that by its size, shape, and appearance (phase-dark) would be expected to exhibit saltatory movement. The number of inclusions showing such movement was usually not more than three per microscopic field. These inclusions were in the same optical section as those showing streaming movement

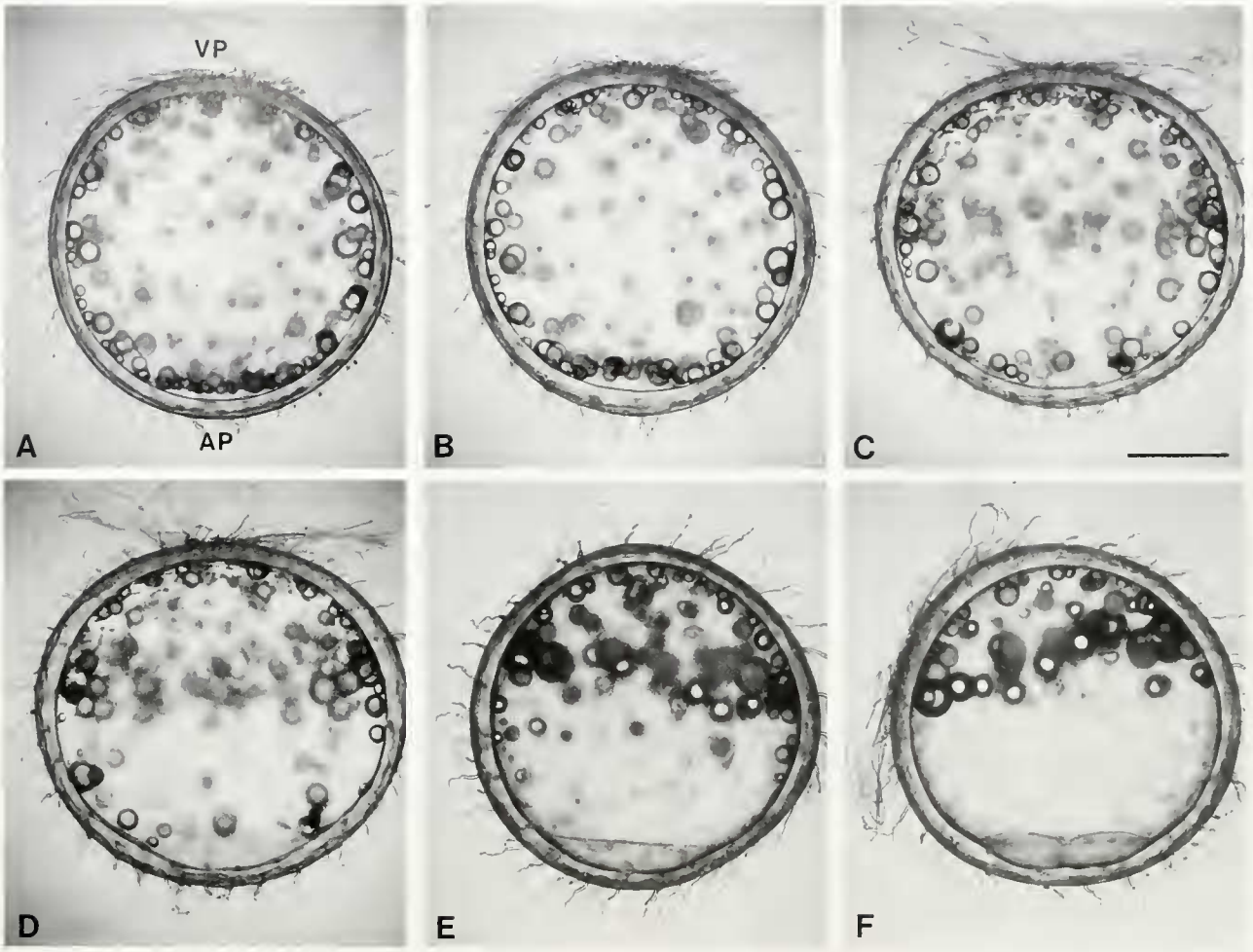

Figure 1. Ooplasmic segregation in the medaka egg. (A) $t_n = 0.07$. The just-fertilized egg consists of a chorion covered with hairs, a large yolk vacuole, and a thin peripheral layer of ooplasm between the yolk membrane and plasma membrane. Oil droplets are present throughout the ooplasm, and a thin blastodisc is visible at the animal pole (AP). (B) $t_n = 0.25$. The thickness of the blastodisc has increased, but oil droplet movement toward the vegetal pole has not yet begun. (C) $t_n = 0.53$. The thickness of the blastodisc has increased even more, and oil droplets have begun to move toward the vegetal pole. (D) $t_n = 0.69$. A biconvex blastodisc has formed, and many oil droplets have left the animal hemisphere. (E) $t_n = 0.81$. The blastodisc has become plano-convex, and oil droplet movement continues. (F) $t_n = 1.00$ (about 70 min at 23°C). The blastodisc has begun to undergo cytokinesis, and most of the oil droplets have formed a crude cap over the vegetal hemisphere. Scale bar, 500 μm.

toward the animal pole. The movement of such inclusions (Fig. 4B) differed from those that streamed in the following ways: (1) Their motion was intermittent, hence the designation "saltatory." An inclusion showing saltatory motion typically moved at a constant rate for 15–120 sec, paused for 5–20 sec and then began moving again. (2) Their velocity (44.4 ± 13.8 μm min^{-1}, $\bar{X} \pm$ S.D., n = 17 inclusions from 9 eggs) was about 20-fold higher than that of streaming inclusions. (3) They moved toward the vegetal pole, not the animal pole. (4) Whereas streaming inclusions appeared to move directly toward the animal pole, the paths of these inclusions, though generally directed toward the vegetal pole, were more zig-zagged.

Movement of oil droplets

Immediately after the second contraction, oil droplets, like saltatory inclusions, began to move toward the vegetal pole (Fig. 1B–E). Unlike saltatory inclusions, however, oil droplets appeared to move directly toward the vegetal pole. Moreover, oil droplets moved more slowly than sal-

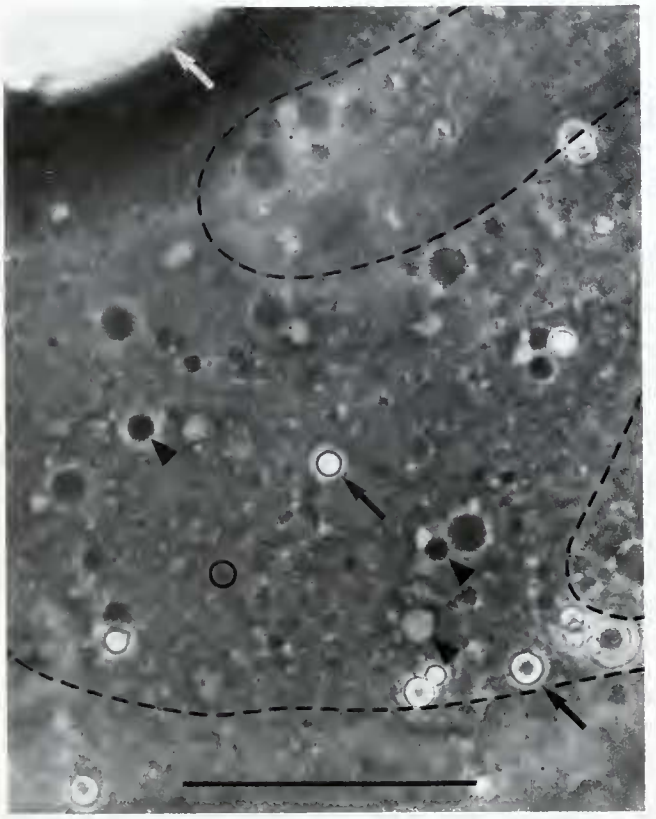

Figure 2. Phase-contrast image of a typical microscopic field near the equator of a fertilized egg at $t_n = 0.43$. Ooplasmic inclusions include some that streamed toward the animal pole (arrowheads), oil droplets of various sizes (arrows), and inclusions that moved saltatorily toward the vegetal pole (encircled parcel). The out-of-focus image of chorionic hairs distorts the image in places (outlined by dashed lines). Scale bar, 50 μm.

tatory inclusions (17.0 ± 5.7 μm min^{-1}, $\bar{X}$ ± S.D., n = 15 droplets in 3 eggs), and their speed varied more than that of saltatory inclusions during a given stretch in which they were moving continuously (Fig. 4C).

Second contraction complex

We monitored this contraction by observing either oil droplet movement at low magnification (at which the entire egg could be seen simultaneously) or the movements of inclusions at higher magnification. In all 18 eggs (from 8 females) in which we analyzed the second contraction, it was composed of at least two components: (1) a movement of ooplasmic inclusions, including oil droplets, toward the vegetal pole, and (2) a subsequent pronounced movement of the inclusions toward the animal pole (Fig. 5A, B). In 5 of the 18 eggs we observed, the movement toward the vegetal pole was preceded by a weaker movement toward the animal pole (data not shown).

Effects of microtubule poisons

Colchicine, colcemid, and nocodazole had the same effects on the eggs, while eggs treated with β-lumicolchicine behaved as untreated (control) eggs. No effect of these poisons was apparent until after the second contraction, even in eggs that were incubated in the microtubule poisons for 1.5 h or 2 h before fertilization: The cortical granule reaction (in 96% of the drug-treated eggs vs. 97% of the controls), the fertilization contraction, elevation of the fertilization membrane, and the second contraction occurred normally in these eggs.

However, these drugs had dramatic effects on the subsequent movement of ooplasmic inclusions toward the poles of the egg. The most obvious effect was on the oil droplets, which floated to the top of the egg instead of moving toward the vegetal pole (Fig. 6B). Moreover, saltatory motion toward the vegetal pole was absent from drug-treated eggs. All three poisons also slowed the rate of growth of the blastodisc (Figs. 6B; 7). The volume of the blastodisc of control eggs at $t_n \approx 0.85$–1.0 was 21.3 ± 4.0 nl ($\bar{X}$ ± S.D., n = 7), while that in eggs treated with microtubule poisons was 11.6 ± 2.6 nl (n = 12). Moreover, in poisoned eggs the blastodisc did not undergo the changes in shape seen in control eggs—from meniscus to biconvex to planoconvex (Fig. 1); instead the blastodisc appeared only to enlarge while maintaining its meniscus form. The microtubule poisons also caused a decrease in the velocity of streaming inclusions [2.2 ± 0.2 μm min^{-1} ($\bar{X}$ ± S.E.M., n = 3 eggs) *versus* 2.9 ± 0.5 (n = 3 eggs) in control eggs]. When we looked at the direction of movement of inclusions during a 10-min period, we found that

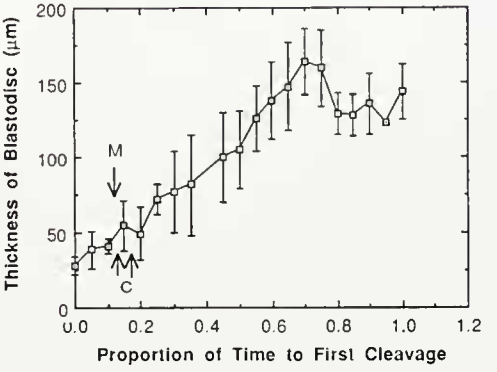

Figure 3. Change in thickness of the blastodisc during ooplasmic segregation. Data from 15 eggs, grown at 14°C–24°C, were used to construct this figure; shown are $\bar{X}$ ± S.D. Arrows mark the times of occurrence of the second meiotic division (M) and the beginning and end of the second contraction (C). The thickness of the blastodisc increased from ≈40 μm in the just-fertilized egg ($t_n = 0.05$) to ≈160 μm at $t_n = 0.7$, by which time the blastodisc was biconvex (Fig. 1D). The decrease in the thickness at $t_n > 0.7$ was caused by a change in the shape of the blastodisc from biconvex to plano-convex before the first mitotic division (Fig. 1D, E).

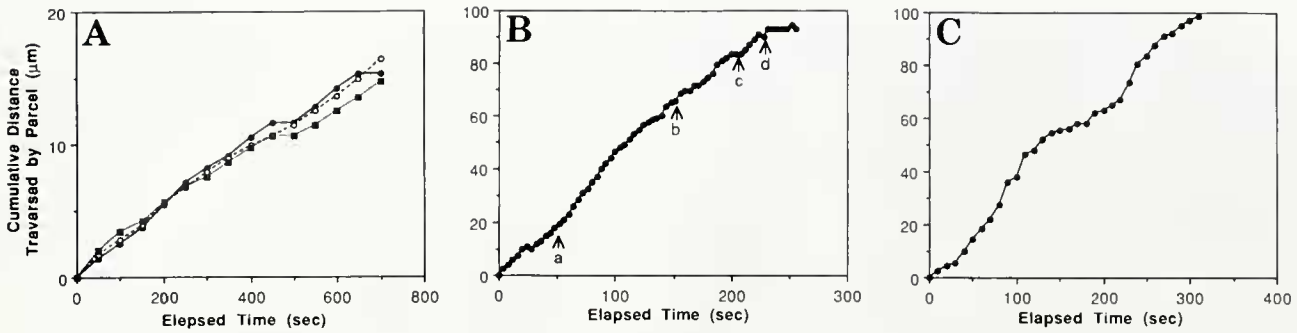

Figure 4. Graphic summary of the movements of ooplasmic inclusions. The movements of five inclusions in a single egg are shown, beginning at $t_n \approx 0.4$. (A) Streaming of inclusions toward the animal pole. Throughout most of this 11+ minute period, the speed of the inclusions was ≈ 1.5 μm min^{-1}. (B) Saltatory motion of an inclusion toward the vegetal pole. Movement was intermittent, *i.e.*, the inclusion sometimes moved rapidly (a → b) and sometimes paused (c). Occasionally such inclusions reversed their direction (d). The velocity of the inclusion between t ≈ 28 s and t ≈ 200 s was ≈25.7 μm min^{-1}. (C) Movement of an oil droplet toward the vegetal pole. The motion summarized here is that of a small oil droplet (diam. ≈6 μm). The speed of the droplet was about 30 μm min^{-1} at 50–100 s and about 9 μm min^{-1} at 125–225 s.

whereas inclusions in control eggs moved in essentially the same direction (3.7° ± 10.6° departure from the animal-vegetal axis, $\bar{X}$ ± S.D., n = 30 inclusions; note the small standard deviation), inclusions in poisoned eggs varied substantially in their direction of movement (10.9° ± 55.7°, $\bar{X}$ ± S.D., n = 52 inclusions; note the large standard deviation).

Hoechst 33258 stained three bodies in control eggs fixed at $t_n = 0.45$. One was inferred to be the second polar body (Fig. 8A) on the basis of its protrusion from the surface of the egg, its size, and the presence of a halo of membrane ruffles around it (Brummett *et al.*, 1985). The other two, the male and female pronuclei, were about 5 μm from each other and 51.8 ± 10.2 μm ($\bar{X}$ ± S.D., n = 9) from the polar body (Fig. 8B, C). As in control eggs, Hoechst 33258 stained three bodies in eggs treated with 100 μ*M* colchicine and fixed at $t_n = 0.45$, one of which was the second polar body (Fig. 8D). However, in contrast to the

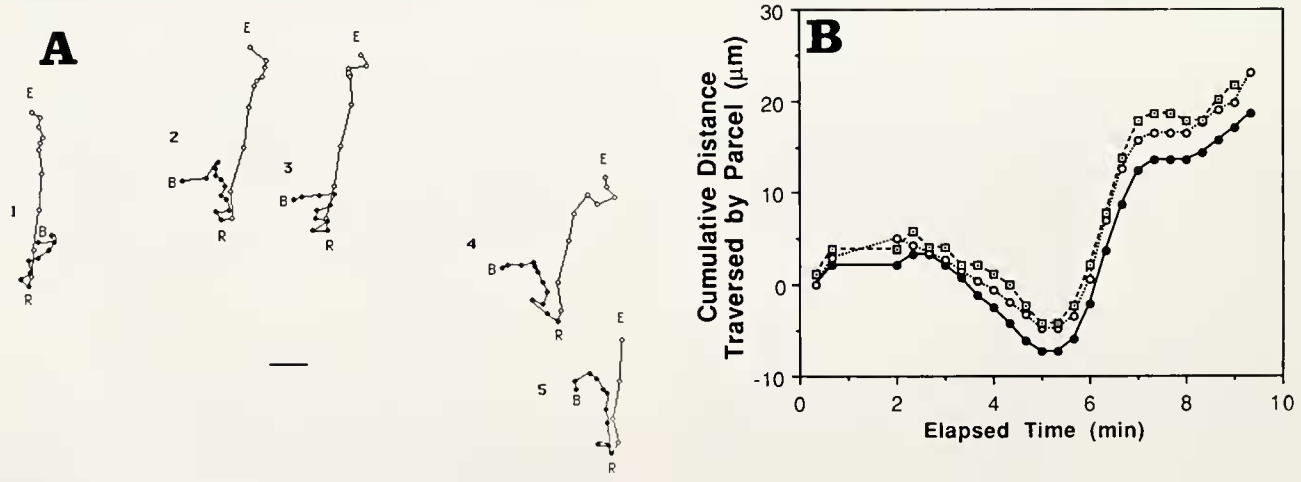

Figure 5. The second contraction. (A) The "tracks" of five inclusions during the second contraction in an egg growing at 21°C. The circles represent the positions of the inclusions at 20 sec intervals, beginning at "B" (6 min after fertilization; $t_n = 0.06$) and ending about 9.5 min later at "E" (at $t_n = 0.15$). The parcels first moved toward the vegetal pole ("down" in this figure; ●—●), and then reversed their direction at "R" and began to move toward the animal pole (o-o). Note that the movement toward the animal pole was rapid at first and then slowed. Scale bar, 5.65 μm. (B) Graphic summary of the movement of parcels #1–3 in Figure 5A. The parcels moved hardly at all for 2 min, began to move toward the vegetal pole (down in this figure) after about 3 min (at $t_n = 0.09$), and then reversed their direction after 5 min ($> t_n = 0.11$) and began to move toward the animal pole (up in this figure).

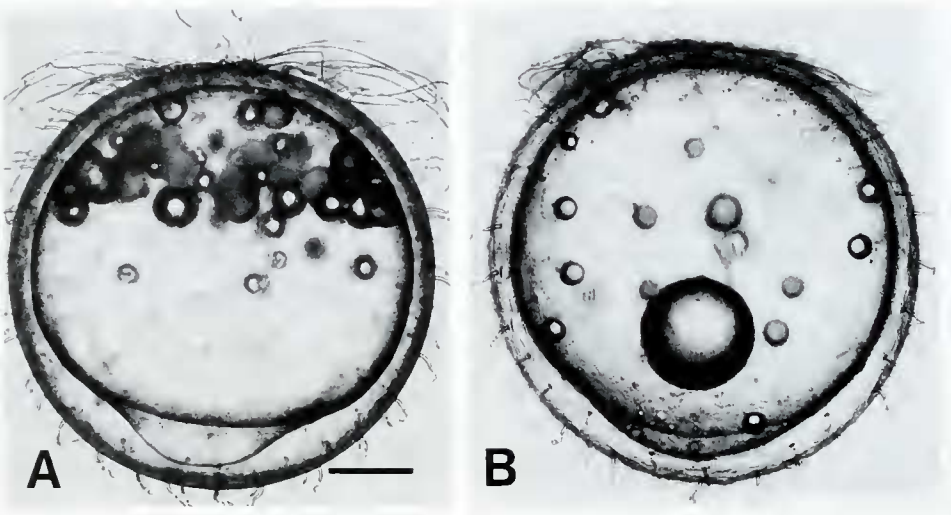

Figure 6. Effects of nocodazole on ooplasmic segregation. (A) Control egg, $t_n = 0.84$. A large blastodisc has formed at the animal pole, and oil droplets have formed a cap over the vegetal hemisphere. (B) Egg treated with 0.17 μM nocodazole, $t_n = 0.80$. The blastodisc is smaller than in the control egg, and most of the oil droplets, instead of moving toward the vegetal pole, have floated to the top of the egg (*i.e.*, toward the viewer, whose perspective is from above the egg) and coalesced into one large droplet there. Scale bar, 250 μm.

situation in control eggs, in which the male and female pronuclei were within 5 μm of each other, the male and female pronuclei in colchicine-treated eggs were far apart (132.2 ± 54.8 μm; $\bar{X}$ ± S.D., n = 8; Fig. 8E).

Discussion

All three microtubule poisons used in this study—colchicine, colcemid, and nocodazole—affected the movement of ooplasmic inclusions during segregation in the medaka egg. That the poisons acted specifically as microtubule poisons is a reasonable inference because (1) their effective concentrations were similar to those used in previous studies (Zalokar, 1974; Eckberg, 1981; Shimizu, 1982; Astrow *et al.*, 1989; Sawada and Schatten, 1989); (2) poisons from two classes of microtubule poisons (Bray, 1992, p. 207) had similar effects on the eggs; and (3) 100 μM β-lumicolchicine had no apparent effect on the eggs. The results of the present study differ from those of Katow (1983), who reported that the blastodisc formed normally in zebrafish eggs treated with colchicine. This difference could be due to the lower concentration of colchicine used in the earlier study (2.5 μM vs. 100 μM in the present study) or to a lower permeability of the zebrafish egg to colchicine. It is possible (but unlikely, we believe) that microtubules are not required for ooplasmic segregation in the zebrafish egg.

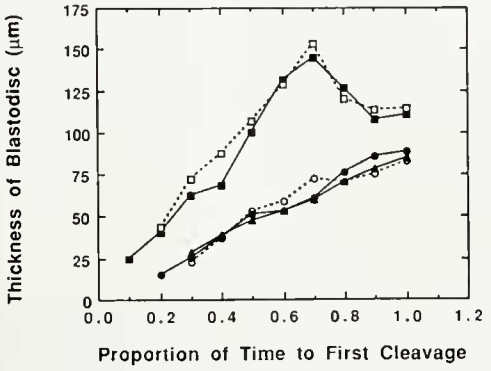

Figure 7. Effect of microtubule poisons on the growth of the blastodisc. The thickness of the blastodisc along the animal-vegetal axis was measured in untreated eggs (— ■ —, four eggs) and in eggs treated with 100 μM β-lumicolchicine (— □ —, three eggs), 100 μM colchicine (— ● —, four eggs), 0.35 μM colcemid (— ○ —, three eggs), or 0.17 μM nocodazole (— ▲ —, five eggs). Shown are the mean values; over all the treatments and times, the standard deviation averaged 12% of the mean. Colcemid, colchicine, and nocodazole all slowed the growth of the blastodisc, while eggs treated with β-lulmicolchicine behaved as untreated eggs. The volume of the blastodisc in control eggs and poisoned eggs at $t_n \approx 0.85$–1.0 was 21.3 ± 4.0 nl and 11.6 ± 2.6 nl ($\bar{X}$ ± SD), respectively.

Saltatory movement similar to that observed in the present study is often associated with microtubules (Hayden *et al.*, 1983; Brady and Pfister, 1991). Similarities include the intermittent nature of the movement, its inhibition by microtubule poisons, and the speed of moving particles (Hamaguchi *et al.*, 1986; Shimizu *et al.*, 1991). These similarities suggest that in the medaka egg some ooplasmic inclusions move toward the vegetal pole via microtubules oriented approximately along the animal-vegetal axis.

The normal movement of oil droplets was also affected by the poisons, suggesting that microtubules are also involved in the movement of these droplets. Such an in-

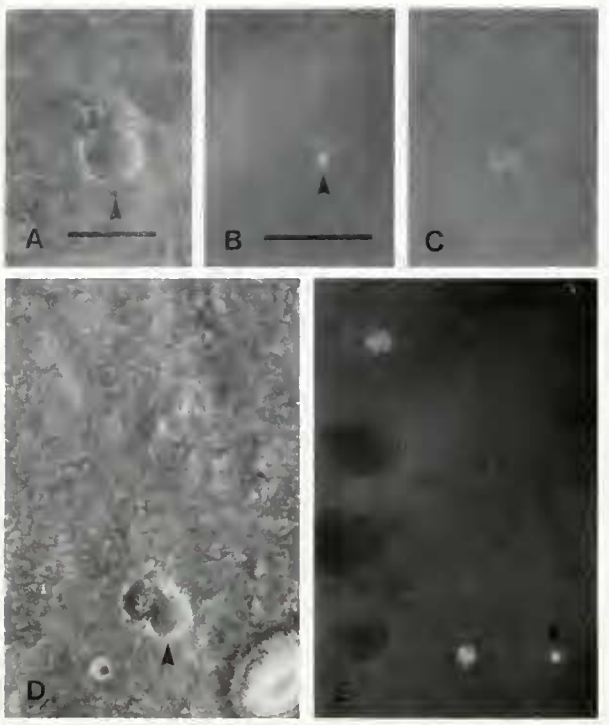

Figure 8. Formation of second polar body and pronuclear migration. Untreated eggs (A–C) and eggs treated with 100 μM colchicine (D–E) were fixed at $t_n = 0.45$, subsequently stained with Hoechst 33258, and viewed with either phase contrast (A, D) or epifluorescence (B, C, E) optics. The second polar body (arrowhead) could be seen near the animal pole in both untreated eggs (A) and in eggs treated with 100 μM colchicine (D). Of the three fluorescent bodies present in this region of the egg, one corresponded to the polar body (arrowhead in B, E), and the other two were the male and female pronuclei (C, E). In the untreated egg shown here, the polar body and pronuclei were recorded in separate photographs (B, C) because their focal planes were separated by about 25 μm. The two pronuclei were close to each other and were about 37 μm from the polar body. In eggs treated with colchicine, the pronuclei were much farther apart (E). Scale bars, A, D, 10 μm; B, C, E, 50 μm.

volvement would seem to require the presence of a unit membrane at the surface of the oil droplets to provide a site of attachment for a kinesin-like molecule. Whether such a membrane is present around these droplets is not known. In other types of cells, unit membranes are present around some lipid droplets but not others (Wake, 1974; Nedergard and Lindberg, 1982). An alternative explanation for the effect of these poisons on oil droplet movement is that in control eggs a dynamic network of microtubules holds the oil droplets in place; in the presence of these microtubule poisons, such a dynamic network would eventually disappear as disassembly continues in the absence of assembly. This question will require further study.

The movement of ooplasm toward the animal pole in fish embryos has been previously described as streaming (Roosen-Runge, 1938; Beams *et al.*, 1985) or bulk flow (Gilkey, 1981); our results confirm these reports. Microtubule poisons slowed both the movement of inclusions toward the animal pole and the growth of the blastodisc, but they did not inhibit either process entirely, suggesting that more than one mechanism is responsible for these phenomena. In ascidians (Sawada and Osanai, 1981, 1984, 1985; Jeffery, 1984; Bates and Jeffery, 1988) and an oligochaete (Shimizu, 1982, 1984), actin microfilaments form a cortical network that contracts toward one pole of the egg, pulling with it both cortical and subcortical components of the ooplasm. F-actin is present in the cortex and subcortex (Beams *et al.*, 1985; Wolenski and Hart, 1987; Chang, 1991) of the zebrafish egg, and an actomyosin-like ATPase has been identified in cortical preparations of fish eggs (Jorgensen, 1972). Moreover, cytochalasins (Katow, 1983; Ivanenkov *et al.*, 1987; Fluck, unpub.) and DNase I (Ivanenkov *et al.*, 1987) inhibit formation of the blastodisc in fish embryos. Thus, both microtubules and microfilaments may be involved in the movement of ooplasm and its inclusions toward the animal pole in the medaka egg.

Calcium ion may both trigger and organize such a contraction in the medaka egg just as it does in ascidian egg (Jeffery, 1982; Sardet *et al.*, 1986; Speksnijder *et al.*, 1990a, b; see also Cheer *et al.*, 1987). Cytosolic $[Ca^{2+}]$ is elevated at the animal and vegetal poles of the medaka egg during ooplasmic segregation (Fluck *et al.*, 1992b), and injection of the weak calcium buffer, dibromo-BAPTA (Speksnijder *et al.*, 1989), into the medaka egg inhibits formation of the blastodisc (Fluck *et al.*, 1992a).

In eggs treated with microtubule poisons, the ooplasm appeared to be solated compared to that in control eggs because oil droplets floated to the top of the egg instead of moving toward the vegetal pole. Moreover, the movement of inclusions toward the animal pole of the egg was more disorganized. Both of these effects could be the result of the disruption of a dynamic network of microtubules in the ooplasm by the microtubule poisons.

All three of the movements described in this report—streaming toward the animal pole, saltatory movement toward the vegetal pole, and movement of oil droplets toward the vegetal pole–began only after formation of the second polar body and the second contraction; this was true for both control eggs and for eggs treated with microtubule poisons. These events thus heralded a radical change in the structure and/or activity of the cytoskeleton of the egg. Though the present study is the first to describe this phenomenon as a contraction (or series of contractions), both Sakai (1965) and Iwamatsu (1973) reported that oil droplets in the medaka egg oscillate along the animal-vegetal axis at this time.

In addition to its effect on ooplasmic segregation, colchicine inhibited the migration of the pronuclei after formation of the second polar body, which apparently formed normally. In its insensitivity to a microtubule poison, the

medaka egg is like that of the ascidian *Phallusia mammillata* (Zalokar, 1974) and the polychaete *Chaetopterus pergamentaceus* (Eckberg, 1981), but unlike that of the leech *Helobdella triserialis* (Astrow *et al.*, 1989) and the oligochaete *Tubifex hattai* (Shimizu, 1982). In contrast to their variable effects on polar body formation, microtubule poisons consistently inhibit pronuclear migration (Zalokar, 1974; Hiramoto *et al.*, 1984; Hamaguchi and Hiramoto, 1986; Sawada and Schatten, 1989). The results of the present study are consistent with these earlier studies.

These results suggest that in the medaka egg microtubules are necessary for the movement of some components of the ooplasm to the vegetal pole and of others to the animal pole. Such movement in an animal egg is generally considered to constitute "ooplasmic segregation," but whether it also constitutes "cytoplasmic localization" (Davidson, 1976) remains to be seen. These movements in the medaka egg, especially the saltatory ones, may simply reflect the need of this large, polarized cell to sustain its polarity in the same way that epithelial cells (Rindler *et al.*, 1987; Achler *et al.*, 1989; Breitfield *et al.*, 1990) and other eggs (Peter *et al.*, 1991) do.

Acknowledgments

Doug Antonioli, Jesse Fluck, and Justine Fluck assisted in the studies summarized in Figure 3; Justine Fluck in the analysis of that data and the preparation of Figure 3; and Minerva Medina in the studies of the timing of the second meiotic division. We thank Dr. Lionel Jaffe and Dr. Andrew Miller for helpful discussions of these phenomena; Dr. Jaffe for helping us improve the text of the manuscript; and Dr. George Rosenstein and Dr. Gene Johnson for their help in measuring the volume of the blastodisc. Supported by NSF DCB-9017210 and Franklin and Marshall College's Hackman Scholar Program and STEP program.

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